

Cell-Free Rolling Mediated by L-Selectin and Sialyl Lewis^x Reveals the Shear Threshold Effect

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ABSTRACT The selectin family of adhesion molecules mediates attachment and rolling of neutrophils to stimulated endothelial cells. This step of the inflammatory response is a prerequisite to firm attachment and extravasation. We have reported that microspheres coated with sialyl Lewis^x (sLe^x) interact specifically and roll over E-selectin and P-selectin substrates (Brunk et al., 1996; Rodgers et al., 2000). This paper extends the use of the cell-free system to the study of the interactions between L-selectin and sLe^x under flow. We find that sLe^x microspheres specifically interact with and roll on L-selectin substrates. Rolling velocity increases with wall shear stress and decreases with increasing L-selectin density. Rolling velocities are fast, between 25 and 225 $\mu\text{m/s}$, typical of L-selectin interactions. The variability of rolling velocity, quantified by the variance in rolling velocity, scales linearly with rolling velocity. Rolling flux varies with both wall shear stress and L-selectin site density. At a density of L-selectin of 800 sites/ μm^2 , the rolling flux of sLe^x coated microspheres goes through a clear maximum with respect to shear stress at 0.7 dyne/cm². This behavior, in which the maintenance and promotion of rolling interactions on selectins requires shear stress above a threshold value, is known as the shear threshold effect. We found that the magnitude of the effect is greatest at an L-selectin density of 800 sites/ μm^2 and gradually diminishes with increasing L-selectin site density. Our study is the first to reveal the shear threshold effect with a cell free system and the first to show the dependence of the shear threshold effect on L-selectin site density in a reconstituted system. Our ability to recreate the shear threshold effect in a cell-free system strongly suggests the origin of the effect is in the physical chemistry of L-selectin interaction with its ligand, and largely eliminates cellular features such as deformability or topography as its cause.

INTRODUCTION

The selectins, consisting of L-, E- and P-selectin, are important cell adhesion molecules that play a major role in the initial stages of the inflammatory response. The selectins work in concert to mediate the capture and rolling of blood borne neutrophils, a class of phagocytic white blood cells, over stimulated vascular endothelial cells. L-selectin is present on all varieties of leukocytes (Griffin et al., 1990). Specifically, it acts as a lymphocyte homing receptor to the lymph node vasculature (Kishimoto et al., 1990) and is capable of supporting tethering and rolling interactions on adherent neutrophils, allowing accumulation of neutrophils near already adherent neutrophils, thereby enhancing neutrophil localization at sites of inflammation (Bargatze et al., 1994; Finger et al., 1996; Alon et al., 1996; Fuhlbrigge et al., 1996). More recently, the selectins have been implicated to play a role in the homing of stem and progenitor cells to the bone marrow (Greenberg et al., 2000; Mazo et al., 1998; Frenette et al., 1998).

Since neutrophil rolling over stimulated endothelial cells is a prerequisite for their firm attachment and subsequent

exiting out of the blood vessel at sites of inflammation (von Andrian et al., 1991; Lawrence and Springer, 1991), obtaining a mechanistic understanding of the rolling phenomena is important. Rolling is defined as the transient interaction between a cell and substrate under fluid flow, where the cell's velocity is significantly lower than the velocity of a noninteracting cell near the surface. The force exerted by molecular bonds between the cell and the surface act against the fluid drag force on the cell to tether the cell to the surface. The tethered cell experiences both a force and torque that causes the cell to rotate and translate forward. New bonds form in the new cell-wall contact region, while bonds at the back edge of the cell dissociate. As a consequence of the retardation caused by the molecular tethers, the cell moves slowly forward, in the direction of flow, at a fraction of the velocity expected for a noninteracting cell. When selectins are present on a surface at a concentration too low to support rolling, they support tethering, in which the cell briefly pauses on the substrate and then resumes a free stream velocity.

In order to mediate rolling adhesion, selectins interact specifically with carbohydrate presenting counter-receptors. A carbohydrate termed sialyl Lewis^x, abbreviated as sLe^x, is both sialylated and fucosylated and has been shown to bind all the selectins in static assays (Phillips et al., 1990; Polley et al., 1991; Foxall et al., 1992). Additional research has shown that sLe^x is capable of binding E-selectin (Alon et al., 1995; Brunk et al., 1996), P-selectin (Rodgers et al., 2000) and L-selectin (Alon et al., 1995) under flow. Although the selectins have been shown to specifically bind to

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sLe^x, they bind with much higher affinity to the protein counterreceptors decorated by these sLe^x structures (Lasky, 1992; Varki, 1994). Various glycoproteins, including glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1; Hemmerich et al., 1995), mucosal addressin cell adhesion molecule-1 (MAdCAM-1; Berg et al., 1993), CD34 (Puri et al., 1995; Baumhueter et al., 1993), and P-selectin glycoprotein ligand-1 (PSGL-1; Spertini et al., 1996; Tu et al., 1999) have been reported to bind L-selectin. CD34, GlyCAM-1, and PSGL-1 have all been shown to contain sulfated, sialylated, and fucosylated carbohydrates related to sLe^x (Sako et al., 1995; Pouyani and Seed, 1995; Rosen and Bertozzi, 1996; Tu et al., 1999).

Several researchers have studied L-selectin mediated rolling in vitro. Transfected cells expressing L-selectin were found to roll on and tether to sLe^x glycolipid substrates (Alon et al., 1995) and CD34-coated surfaces (von Andrian et al., 1995). Rolling of neutrophils and T lymphocytes, which both express L-selectin, over peripheral node addressin (PNAd) and CD34 substrates has also been observed (Finger et al., 1996; Puri et al., 1997; Lawrence et al., 1997). Fuhlbrigge and coworkers (1996) observed rolling and tethering of neutrophils, monocytes, and myeloid and lymphoid cell lines on immobilized L-selectin and found that L-selectin ligand activity correlated with sLe^x expression. Recently, it has been shown that a sLe^x-like carbohydrate determinant is responsible for up to 99% of neutrophil rolling on, or attachment to, adherent cells expressing L-selectin (Tu et al., 1999). Although a great deal of information about L-selectin-mediated rolling has been obtained over the last few years, there is still much to learn about the molecular functional requirements of L-selectin ligands. For example, the term "sLe^x-like" in the above description implies that the carbohydrate ligands for L-selectin express sLe^x, but in reality are quite diverse. Each ligand provides a different protein scaffold for the carbohydrate and many copies of sLe^x-bearing carbohydrates may be on each ligand. Furthermore, not all the sLe^x-bearing ligands have been identified on all cells. In addition, carbohydrates closely related to sLe^x show cross-reactivity for E- and P-selectin (Brunk and Hammer, 1997; Rodgers et al., 2000). Given the complexity of these interactions, we need tools to accurately measure and assess the activity of carbohydrate-mediated rolling.

In vitro and in vivo rolling experiments have revealed a shear threshold effect, in which the maintenance and promotion of rolling interactions involving selectins requires shear stress above a threshold value. The shear threshold effect manifests itself as an increase in rolling flux with increasing wall shear stress. Typically, the rolling flux increases up to a maximum, after which the rolling flux decreases with increasing wall shear stress. This behavior was first observed by Finger and coworkers (1996) for T lymphocytes over PNAd or CD34 and for neutrophils on PNAd, but not on E- or P-selectin substrates. The shear

threshold effect has been confirmed for T lymphocytes over PNAd (Lawrence et al., 1997) and CD34 (Puri et al., 1998). The range of shear stresses at which rolling flux is a maximum is between 0.7 and 1.0 dynes/cm². The mechanism behind the shear threshold effect may also have implications for cell-cell accumulation at sites of inflammation. A minimum shear stress (0.7 dynes/cm²) was found to be necessary to promote secondary neutrophil accumulation through tethering to neutrophils already rolling on E-, P-, and L-selectin substrates (Alon et al., 1996). To date, the only evidence of a shear requirement for rolling on E- or P-selectin has come from Lawrence and colleagues (1997) who observed a maximum in the rolling flux of HL-60 cells on E- and P-selectin at 0.25 to 0.5 dynes/cm². In vivo evidence of the shear threshold effect has also been observed for leukocytes in murine venules (Lawrence et al., 1997; Finger et al., 1996). The physiological significance of the shear threshold effect is uncertain, although it can be speculated that it provides a hydrodynamic switch to modulate either the initial attachment or accumulation of cells. It has also been hypothesized that the shear threshold requirement may help prevent inappropriate aggregation of leukocytes and interaction with the vessel wall in vessels with inherently low wall shear stresses, or in hypoperfusion (Alon et al., 1996; Finger et al., 1996).

Given the complexity of selectin-mediated adhesion, cell-free systems are an ideal method for studying receptor-ligand interaction under flow, since interactions between two specific molecules can be observed without the confounding influence of rheology, roughness, signaling and complex molecular display inherent with using cells. Recently, using a parallel plate flow chamber, we showed that sLe^x coated microspheres rolled specifically over E- and P-selectin-IgG chimeric substrates (Brunk et al., 1996; Brunk and Hammer, 1997; Rodgers et al., 2000). These studies qualitatively captured the dynamics of rolling observed with in vitro cellular assays, in that particle rolling velocity was found to be a function of wall shear stress and selectin site density. We also observed fluctuations in the rolling velocity with time that have been demonstrated for neutrophil/endothelial systems (Kaplanski et al., 1993; Goetz et al., 1994). Based on these observations, we suggested sLe^x as a minimal functional binding element for E- and P-selectin, because we could mimic the dynamics of neutrophil rolling with sLe^x-selectin interactions.

The minimum functional binding element required for L-selectin mediated rolling and the mechanism mediating the shear threshold effect remains unclear. Therefore, we utilize our previously developed cell-free system to explore L-selectin interaction with sLe^x under flow. In these experiments, 10 μ m diameter polystyrene latex microspheres are coated with sLe^x, whereas L-selectin-IgG chimera is adsorbed to silanated glass microscope slides. sLe^x-coated microspheres are perfused over the L-selectin substrates at varying wall shear stresses, and rolling flux and average and

instantaneous rolling velocities are determined. As a result of these investigations, we find that sLe^x-coated microspheres interact specifically with L-selectin substrates. Rolling velocities and rolling fluxes are a function of wall shear stress and L-selectin site density. In addition, the shear threshold effect is clearly exhibited by this cell-free system, but is dependent on L-selectin site density. At low densities of L-selectin (approximately 800 sites/ μm^2), microsphere rolling flux over L-selectin substrates goes through a maximum at 0.7 dyne/cm². As the density of L-selectin increases, the shear threshold effect gradually diminishes, and is not exhibited on high density (1500–2000 sites/ μm^2) L-selectin substrates.

MATERIALS AND METHODS

Microspheres

NeutrAvidin (Pierce, Rockford, IL) microspheres were prepared as described earlier (Brunk et al., 1996; Brunk and Hammer, 1997). Briefly, NeutrAvidin was covalently attached to 10- μm diameter carboxylated polystyrene latex microspheres (Polysciences, Inc., Warrington, PA) using water soluble carbodiimide. Any remaining active sites on the microspheres were blocked with 0.2 M ethanolamine, followed by a 1% solution of bovine serum albumin (BSA; Sigma, St. Louis, MO) in phosphate buffered saline (PBS) supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ (PBS+, pH 7.4, solution sterile filtered).

Biotinylated sLe^x was obtained from GlycoTech (Rockville, MD). This carbohydrate probe is approximately 30 kD and consists of sLe^x incorporated into a polyacrylamide matrix substituted with biotin. The probe is multivalent, with a biotin:sLe^x ratio of 1:4. The binding properties of biotin and avidin were utilized to attach biotin-sLe^x to NeutrAvidin microspheres. 100 μl of 0.5 $\mu\text{g}/\text{ml}$ biotin-sLe^x in PBS+ was added to 10⁶ NeutrAvidin beads and incubated for 45 min with occasional vortexing for use with mouse L-selectin substrates.

Streptavidin microspheres (10 μm diameter polystyrene latex) were obtained from Bangs Laboratories (Fishers, IN) and coated with 0.5 $\mu\text{g}/\text{ml}$ biotin-sLe^x as described above. There was no difference in measured rolling velocities for the sLe^x coated streptavidin and NeutrAvidin microspheres over mouse L-selectin (data not shown). We conclude that these microspheres behave similarly and therefore some data presented include both microsphere types in the average. For experiments with human L-selectin, 10.9 μm diameter streptavidin microspheres from Bangs Laboratories were coated with 1.0 $\mu\text{g}/\text{ml}$ biotin-sLe^x as described above.

The presence of sLe^x on the microspheres was confirmed by flow cytometry as described previously (Brunk and Hammer, 1997). Briefly, a primary monoclonal antibody to sLe^x (30 $\mu\text{g}/\text{ml}$, SNH4, mouse IgG₃, gift from Dr. Anil Singhal, Biomembrane Institute, Seattle, WA) in morpholinoethanesulfonic acid (MES) buffer, pH 5.5, 1 mM CaCl₂, 1 mM MgCl₂, 1% BSA, sterile filtered) was added to sLe^x-coated microspheres and allowed to incubate at room temperature for 45 min. After removing the primary antibody solution, a fluorescent monoclonal secondary antibody, specific for mouse IgG₃ (fluorescein isothiocyanate (FITC) anti-mouse IgG₃, rat IgG_{2a}, Pharmingen, San Diego, CA) was added at a concentration of 60 $\mu\text{g}/\text{ml}$ and allowed to incubate at room temperature for 45 min before flow cytometry analysis. The maximum surface density of sLe^x on the microspheres was found to be 90 molecules/ μm^2 , with saturation of the microsphere surface occurring at 0.3 $\mu\text{g}/\text{ml}$ sLe^x incubation concentration (Brunk and Hammer, 1997).

L-Selectin substrates

Human L-selectin-IgG chimera was a gift from Dr. Ray Camphausen (Genetics Institute, Cambridge, MA). Mouse L-selectin chimera was a gift

from Dr. Brian Brandley (Rush Medical Center, Chicago, IL). The chimeras consist of the lectin, epidermal growth factor, and multiple short consensus repeat domains for human or mouse L-selectin linked to the Fc region of human IgG. L-selectin chimera was adsorbed to silanated glass microscope slides. Briefly, the desired concentration of L-selectin chimera (0.5–5.0 $\mu\text{g}/\text{ml}$) in PBS, pH 7.4, was incubated on silanated glass microscope slides (Sigma) using modified flexiPERM wells (Sigma) for at least 2 h at room temperature with gentle mixing. Slides were then washed with PBS and incubated with blocking buffer for at least 1 h at room temperature to prevent nonspecific adhesion. For experiments with human L-selectin, blocking buffer was PBS containing 2% BSA that was heated at 56°C for 30 min before blocking to denature the BSA. With mouse L-selectin, the slides were incubated in PBS+ for at least 1 h at 37°C to prevent nonspecific adhesion.

L-Selectin site density estimates were obtained using a FITC-labeled monoclonal antibody to mouse L-selectin, Mel-14 (rat IgG_{2a}κ, Pharmingen) or human L-selectin, DREG56 (mouse IgG₁, Caltag, Burlingame, CA) and measuring the fluorescence in counts/s with a Nikon Diaphot inverted microscope (Melville, NY) equipped with a fluorescein cube and connected to a photomultiplier tube (Photocan, Nikon). The construction of the calibration curve to convert fluorescence into site densities has been described previously (Brunk and Hammer, 1997). A graph of estimated L-selectin molecules per area as a function of bulk mouse or human L-selectin chimera concentration during incubation is shown in Fig. 1, with error bars representing 95% confidence intervals. These estimates are based on the assumption that there is 1:1 binding between the antibodies and L-selectin.

Flow chamber

All experiments were conducted in a parallel plate flow chamber with a tapered channel design that allows for a linear variation of shear stress down the length of the flow channel at a single flow rate and channel height. This design is ideal for these experiments, as it allowed us to measure adhesion at many different shear stresses in a single experiment. The design is based on Hele-Shaw flow theory between parallel plates and has been previously described (Usami et al., 1993). The plates were separated by 250 μm Duralastic sheeting (Allied Biomedical, Paso Robles,

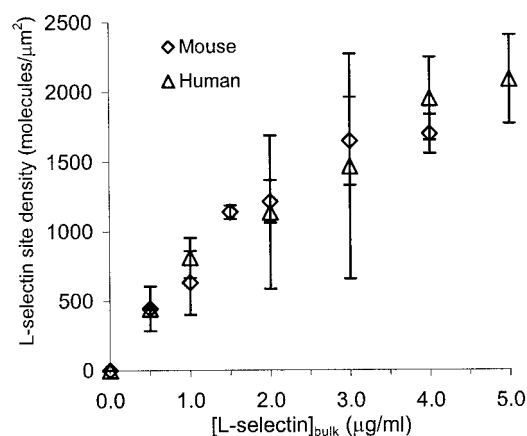


FIGURE 1 Site density of L-selectin chimera molecules on silanated glass slides as a function of bulk incubation concentration. This graph suggests that the slide surface is saturated with mouse and human L-selectin chimera for bulk concentrations greater than 3.0 or 4.0 $\mu\text{g}/\text{ml}$, respectively. Slides were incubated with up to 4.0 $\mu\text{g}/\text{ml}$ mouse L-selectin-IgG chimera or 5.0 $\mu\text{g}/\text{ml}$ human L-selectin-IgG chimera. Points are the average of two to four independent fluorescence measurements. Error bars are 95% confidence intervals.

CA), which compressed to 180 μm when the flow chamber was fully assembled and tightened. The selectin-coated microscope slides serve as the bottom plate of the flow chamber. During experiments, the chamber was secured on the stage of a Nikon Diaphot inverted phase contrast microscope connected to a monochrome CCD video camera (Cohu, Inc., San Diego, CA) and an S-VHS videocassette recorder (Model SVO-9500MD; Sony Electronics, Park Ridge, NJ). Buffer and cell suspensions were drawn through the chamber by an infusion/withdrawal syringe pump (Harvard Apparatus, South Natick, MA).

Adhesion experiments

Selectin-coated slides were placed in the well of the flow chamber, which was assembled in PBS to prevent air bubbles and then secured on the microscope stage. The chamber was perfused with PBS+, and carbohydrate-coated microspheres at a concentration of $5 \times 10^5/\text{ml}$ in PBS+ were then introduced into the flow channel. Data were collected by stepping down the chamber from inlet to outlet in 5 mm steps, allowing at least 1 min between steps. Cell interaction with the surface was recorded for future analysis at a total magnification of $300\times$ (using a $10\times$ objective). All experiments were done at room temperature.

Antibody blocking experiments were performed similar to the experiments described above, except L-selectin slides were incubated with 20 $\mu\text{g}/\text{ml}$ DREG56 (anti-human L-selectin, mouse IgG₁, gift from Dr. Kei Kishimoto, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) or Mel-14 (FITC anti-mouse L-selectin, rat IgG_{2a}, Pharmingen) in PBS for 30 min at room temperature and then placed in the chamber. Control antibody experiments were done similarly with anti-human IgG₁ (mouse IgG₁, Pharmingen) or anti-mouse IgG₃ (FITC, rat IgG_{2a}, Pharmingen). For experiments with EDTA or fucoidan, the slides were incubated for 15 min in 5 mM EDTA or 10 $\mu\text{g}/\text{ml}$ fucoidan in PBS. The microsphere suspension was also supplemented with these concentrations of EDTA or fucoidan.

Data analysis

Velocity measurements were obtained from recorded data using National Instruments (Austin, TX) image acquisition (IMAQ) PCI-1408 frame grabber board, IMAQ software, and LabVIEW 4.0. Virtual instruments (VIs) used in LabVIEW were developed to determine rolling velocities and have been described previously (Greenberg et al., 2000). Briefly, VIs automatically advanced the VCR a specified number of frames, grabbed a designated number of frames spaced equally apart, converted each captured frame into a binary image according to user supplied criteria, detected cells on each image according to user-input criteria, and recorded these cell positions as coordinates on a two-dimensional array. Another VI was then used to plot cell trajectories by using the coordinate arrays from the images and representing each detected cell from each grabbed frame as a point on a background plot. Trajectories were then manually selected, and instantaneous and average velocities in both the x - and y -directions along with the standard deviation of the average velocities were automatically calculated and sent to a tab-delimited text file that could be imported into spreadsheet and graphing programs. Instantaneous velocity was calculated by dividing the displacement of a rolling cell by the time between incremental captured frames. Average velocity was calculated by averaging the instantaneous velocities for a given trajectory. The time between incremental captured frames was set between 0.10 s (3 frames) to 0.33 s (10 frames) depending on how fast the microspheres were rolling. A minimum of 20 iterations was completed to obtain a trajectory.

In order to determine the rolling flux (number of microspheres rolling/ min/mm^2), the number of rolling microspheres in each field of view, consisting of a 0.32 mm^2 area, were counted for 1 min at each shear stress. Microspheres were counted if they rolled for >10 cell diameters while remaining in the field of view. However, at certain shear stresses and L-selectin surface densities, microspheres alternate between rolling and

moving at the free stream hydrodynamic velocity. Therefore, in order to objectively count a microsphere as rolling, it had to move at least 50% slower than the calculated free stream velocity of a noninteracting microsphere. Free stream velocities were calculated using the theory of Goldman, Cox, and Brenner (Goldman et al., 1967a,b) for a 10.9 μm diameter sphere at a particle to surface separation of 50 nm and range from 105 $\mu\text{m}/\text{s}$ at a wall shear stress of 0.4 dynes/cm^2 to 664 $\mu\text{m}/\text{s}$ at a wall shear stress of 2.45 dynes/cm^2 . At this particle-to-surface separation, it is assumed that a cell would be able to interact with and roll on the selectin-coated surface.

Firm attachment flux was measured by counting the number of non-moving microspheres at the end of each recording period. Nonmoving particles were defined as spheres remaining stationary on the surface for at least 10 s. This number was then divided by the total time elapsed during the experiment (up to the total measurement time) and the area of the viewing window. Firm adhesion was insignificant in experiments done with human L-selectin.

RESULTS

sLe^x interaction with L-selectin substrates under flow

Polystyrene microspheres coated with sialyl Lewis x (sLe^x) interact specifically with L-selectin substrates under flow. The site density of mouse or human L-selectin-IgG chimera adsorbed to silanated glass microscope slides as a function of bulk L-selectin incubation concentration is shown in Fig. 1. The graph suggests that the slide surface is saturated with mouse or human L-selectin chimera for bulk concentrations greater than 3.0 or 4.0 $\mu\text{g}/\text{ml}$, respectively. The lectin domain of mouse L-selectin is 86% homologous to human L-selectin (Siegelman and Weissman, 1989) and all other structural domains are conserved. Because the structures of mouse and human L-selectin chimeras are so similar, it is not surprising that the chimeras adsorb in a comparable manner to silanated glass microscope slides. Small discrepancies in site density determinations between mouse and human L-selectin chimera could be due to differences in the fluorescent antibodies used for site density measurements (see Methods).

Fig. 2 compares rolling and firm attachment fluxes for sLe^x microspheres over L-selectin substrates at a wall shear stress of 0.6 dynes/cm^2 on mouse L-selectin (Fig. 2 *A*) and 1.05 dynes/cm^2 on human L-selectin (Fig. 2 *B*). Microspheres were counted as rolling if they rolled for >10 cell diameters while remaining in the field of view or moved at a velocity at least 50% slower than the free stream velocity of a noninteracting microsphere (see Methods). Absence of 95% confidence level error bars means that the experimentally determined particle flux is zero. Various molecules were added to verify rolling specificity. Addition of Mel-14, a function-blocking antibody specific for the lectin domain of mouse L-selectin (Gallatin et al., 1983), completely blocks rolling on mouse L-selectin. Similarly, addition of DREG56, a function-blocking antibody specific for the lectin domain of human L-selectin (Kishimoto et al., 1990), also blocks rolling on surfaces of human L-selectin. Fucoidan, a sulfated polymer of fucose that specifically binds to the lectin domain of L-selectin and inhibits adhesion

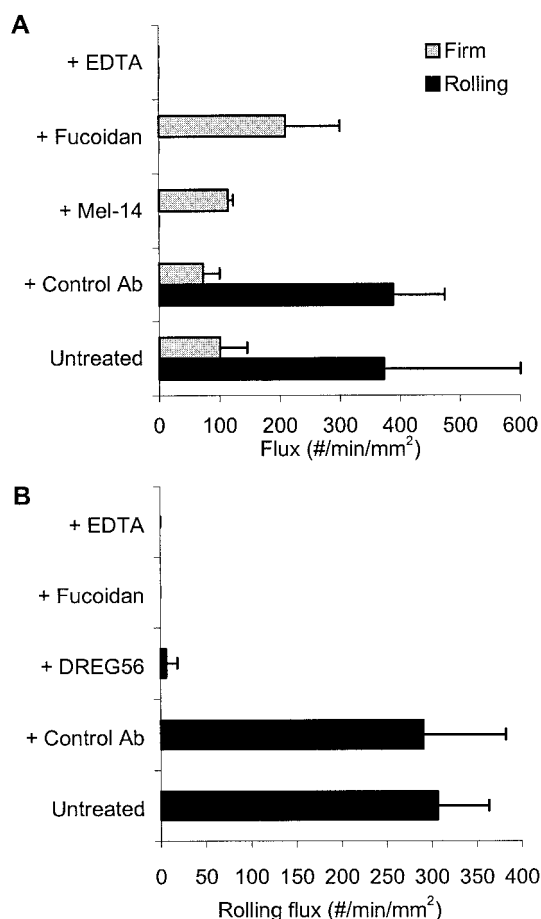


FIGURE 2 Rolling and firm attachment fluxes for sLe^x beads over mouse and human L-selectin chimera substrates. (A) Addition of Mel-14 (10 μ g/ml), fucoidan (10 μ g/ml), or EDTA (5 mM) each blocked microsphere rolling on the mouse L-selectin chimera surface, whereas a control antibody had no effect. All fluxes were obtained at a wall shear stress of 0.6 dynes/cm². Microspheres were prepared with 0.5 μ g/ml biotinylated sLe^x. A concentration of 2.5 μ g/ml L-selectin chimera was added to the slides during incubation. (B) Addition of DREG56 (20 μ g/ml), fucoidan (10 μ g/ml), or EDTA (5 mM) each blocked microsphere interaction with the human L-selectin chimera surface, whereas a control antibody had no effect. All fluxes were obtained at a wall shear stress of 1.05 dynes/cm². Microspheres were prepared using 1.0 μ g/ml biotinylated sLe^x and a concentration of 5.0 μ g/ml L-selectin chimera was added to the slides during incubation. Mean flux for all experiments is the result of averaging from three to six independent experiments. Where bars are not visible, flux is zero. Error bars represent 95% confidence intervals.

(Foxall et al., 1992), completely inhibits rolling of sLe^x-coated particles on both mouse and human L-selectin. EDTA, when added at a concentration of 5 mM, blocks sLe^x interaction with both types of L-selectin substrates, suggesting that the transient interaction is cation-dependent, consistent with the known Ca²⁺ requirement of the selectins. Addition of an isotype-matched antibody in place of Mel-14 (FITC anti-mouse IgG₃, rat IgG_{2a}) or DREG56 (anti-human IgG₁, mouse IgG₁) has no effect on rolling flux or rolling velocity (data not shown). These results demonstrate that

sLe^x is able to mediate rolling on L-selectin in a cell-free system and that the interaction between sLe^x coated beads and L-selectin substrates is specific.

The number of particles adherent to the surface for at least 10 s is represented by the firm attachment flux in Fig. 2 A. The level of firm attachment on mouse L-selectin remains relatively constant for the fucoidan, Mel-14, control antibody, and untreated systems. Addition of EDTA completely abolishes firm attachment on mouse L-selectin. Taken together, these observations suggest that firm attachment is nonspecific for the lectin region of mouse L-selectin chimera, where functional binding occurs, and is enhanced by the presence of divalent cations in the buffer. Although the background nonspecific firm binding flux is much less than the rolling flux on mouse L-selectin for the untreated system, we decided to investigate if nonspecific binding could be eliminated by using a human L-selectin chimera. With human L-selectin and a change in blocking buffer from 1% BSA in PBS to 2% denatured BSA in PBS (see Methods), virtually no firm attachment to L-selectin is observed. Thus, we have used experiments on both mouse and human L-selectin to identify the mechanisms of L-selectin-mediated rolling.

Effect of wall shear stress on rolling interactions with mouse L-selectin

The average particle rolling velocity and rolling flux as a function of wall shear stress for sLe^x-coated microspheres over 2.5 μ g/ml (\sim 1400 sites/ μ m²) mouse L-selectin chimera is shown in Fig. 3. Average particle rolling velocity increases with increasing wall shear stress (Fig. 3 A). Average rolling velocity increases eightfold with a fivefold increase in wall shear stress. An increase in rolling velocity with shear stress has also been observed for T lymphocytes interacting with CD34 coated substrates (Puri and Springer, 1996) and for neutrophils interacting with substrates expressing human L-selectin (Fuhlbrigge et al., 1996). Additionally, the rolling velocities we observe are of the same order of magnitude as those reported at similar wall shear stresses for L-selectin-transfected L1-2 cells (a murine cell line) over PNAd substrates (von Andrian et al., 1995) and in mouse venules (Stein et al., 1999). These observations suggest that our simplified cell-free system captures the essential dynamics of rolling on L-selectin.

sLe^x microspheres show a maximum in rolling flux at 1.0 dyne/cm² on mouse L-selectin (Fig. 3 B). With a threefold increase in wall shear stress above 1.0 dyne/cm², rolling flux decreases 75%. However, at wall shear stresses below 1.0 dyne/cm², rolling flux decreases with a decrease in shear stress and is significantly lower than the flux measured at 1.0 dyne/cm² (at a 95% confidence level). These results reveal the presence of the shear threshold effect with the sLe^x/mouse L-selectin cell-free system.

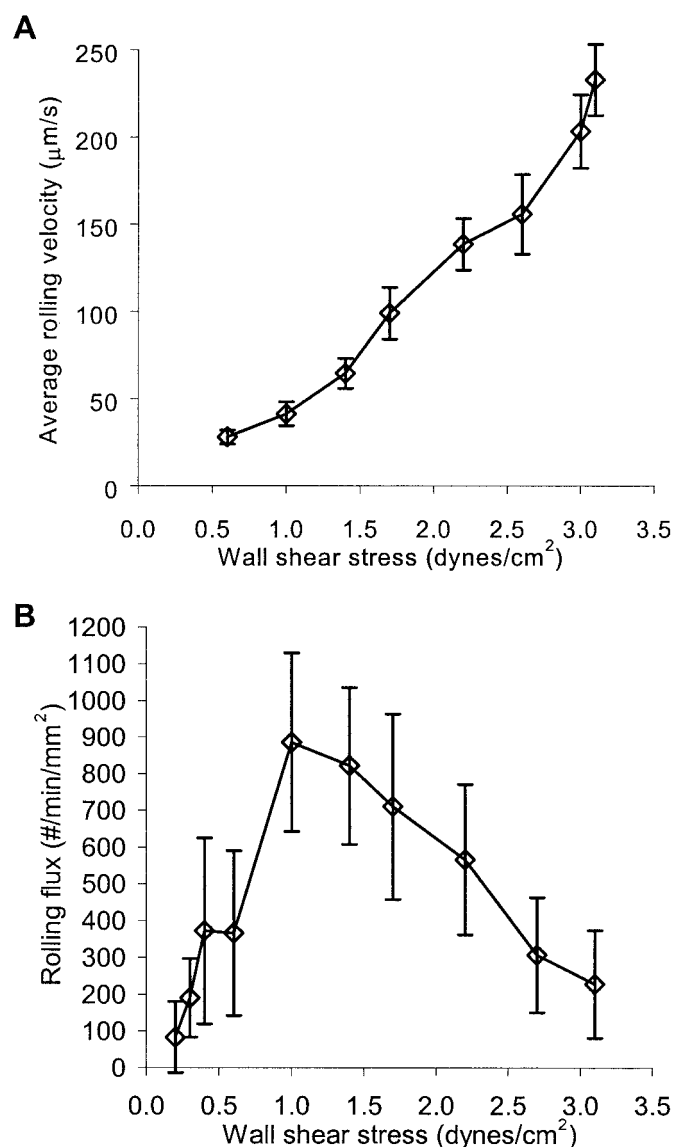


FIGURE 3 Rolling of sLe^x microspheres on mouse L-selectin chimera. sLe^x beads interact with mouse L-selectin substrates maximally at 1 dynes/cm^2 . (A) Average particle rolling velocity as a function of wall shear stress. (B) Rolling flux as a function of wall shear stress. Microspheres were prepared with 0.5 $\mu\text{g/ml}$ biotinylated sLe^x. A concentration of 2.5 $\mu\text{g/ml}$ L-selectin-IgG was added to the slides during incubation. Results are averages obtained from six independent experiments. Error bars are 95% confidence levels.

Effect of L-selectin site density changes and wall shear stress on rolling velocity

In order to study the effect of L-selectin site density on particle rolling velocities and rolling fluxes, we perfused sLe^x coated microspheres over human L-selectin substrates that had been incubated with 0.5 to 5.0 $\mu\text{g/ml}$ L-selectin chimera. From our site density calculations (Fig. 1), incubation of 0.5 to 5.0 $\mu\text{g/ml}$ L-selectin results in L-selectin site densities of approximately 400 to 2000 sites/ μm^2 . Fig.

4 A compares representative plots of position as a function of time for sLe^x-coated microspheres rolling over saturating and reduced concentrations of human L-selectin at a wall shear stress of 2.05 dynes/cm^2 . As L-selectin site density decreases, rolling becomes less smooth, as indicated by more frequent changes in the slope of each line, and eventually reaches the tethering limit. At 1.0 $\mu\text{g/ml}$ (800 sites/ μm^2) L-selectin incubation, rolling microspheres alternate between fast and slower movement, and average rolling

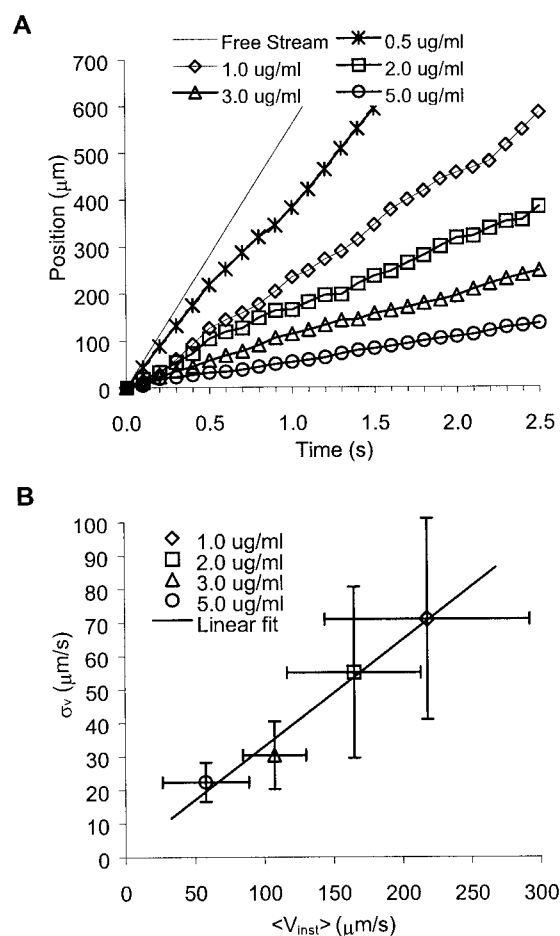


FIGURE 4 Position and instantaneous rolling velocity of sLe^x coated microspheres rolling over saturating and reduced concentrations of human L-selectin at 2.05 dynes/cm^2 . As L-selectin site density decreases, rolling becomes less smooth and eventually reaches the tethering limit. Microspheres were prepared using 1.0 $\mu\text{g/ml}$ biotinylated sLe^x and a concentration of 0.5 to 5.0 $\mu\text{g/ml}$ L-selectin-IgG was added to slides during incubation. Position and instantaneous rolling velocity data were acquired at a rate of 10 per second. (A) Representative plots of position of rolling sLe^x microspheres as a function of time. The plot for a noninteracting microsphere moving at the calculated free stream velocity (see Methods) is included for comparison. (B) Ensemble-averaged standard deviation of instantaneous rolling velocities, σ_v , as a function of ensemble-averaged instantaneous rolling velocity, $\langle V_{\text{inst}} \rangle$, and L-selectin site density. Each data point represents mean values from 10 rolling microspheres with instantaneous rolling velocities calculated for at least 2 s for microsphere. The R^2 value for the linear fit of the mean data is 0.98. Error bars are standard deviations of mean values.

velocity approaches 50% of the free stream velocity of a noninteracting microsphere, defined previously as the limit for rolling behavior (see Methods). At an incubation concentration of 0.5 $\mu\text{g/ml}$ (400 sites/ μm^2), microspheres exhibit tethering behavior, in which they briefly pause, but do not steadily roll, on the L-selectin substrate. The effect of site density on rolling behavior for a population of sLe^x microspheres is quantified in Fig. 4 B. Instantaneous rolling velocities, V_{inst} , were obtained using position data acquired at a rate of 10/s. The ensemble-averaged instantaneous rolling velocity, $\langle V_{\text{inst}} \rangle$, is calculated with data from 10 rolling sLe^x microspheres for each L-selectin site density over a total time period of at least 2.5 s. The variability in rolling velocity can be quantified using the ensemble-averaged standard deviation of the instantaneous rolling velocity, σ_v . With a decrease in L-selectin site density, both $\langle V_{\text{inst}} \rangle$ and σ_v increase. The correlation of σ_v with $\langle V_{\text{inst}} \rangle$ is linear, with an R^2 of 0.98. A similar linear correlation has been observed previously for sLe^x microspheres rolling over E-selectin substrates (Brunk and Hammer, 1997). The variance in both $\langle V_{\text{inst}} \rangle$ and σ_v , as represented by standard deviation error bars, also increases with a decrease in L-selectin site density. Thus, larger velocity fluctuations are evident as L-selectin site density decreases.

Fig. 5 compares the average particle rolling velocity as a function of wall shear stress for saturated and reduced levels of L-selectin. Mean rolling velocities on human L-selectin substrates range from 30 $\mu\text{m/s}$ at 0.4 dynes/ cm^2 (5.0 $\mu\text{g/ml}$ L-selectin, 2000 sites/ μm^2) to 220 $\mu\text{m/s}$ at 2.05 dynes/ cm^2 (1.0 $\mu\text{g/ml}$, 800 sites/ μm^2). As a comparison, the average rolling velocity of neutrophils on 2.0 $\mu\text{g/ml}$ L-selectin chimera at 1 dyne/ cm^2 was found to be 35 $\mu\text{m/s}$ (Fuhlbrigge et

al., 1996), and on 0.3 $\mu\text{g/ml}$ purified L-selectin at 2 dynes/ cm^2 , average neutrophil rolling velocity was 100 $\mu\text{m/s}$ (Alon et al., 1998). Therefore, the magnitude of the average rolling velocities of sLe^x microspheres on human L-selectin compares well with the average rolling velocity of neutrophils on human L-selectin over a similar range of wall shear stresses.

Decreases in L-selectin density increase average rolling velocities across all the wall shear stresses observed (Fig. 5). However, this increase in rolling velocity is greatest at higher wall shear stresses. With a 60% decrease in L-selectin density (from 2000 to 800 sites/ μm^2 or 5.0 to 1.0 $\mu\text{g/ml}$), average rolling velocity increases 1.5-fold to threefold. Average rolling velocity increases with wall shear stress up to 2.05 dynes/ cm^2 on saturated and reduced levels of L-selectin. With a fivefold increase in wall shear stress on human L-selectin (from 0.4 to 2.05 dynes/ cm^2), average rolling velocity increases three- to fivefold, depending on L-selectin density. The greatest increase with wall shear stress occurs on the lowest density of L-selectin (800 sites/ μm^2 , 1.0 $\mu\text{g/ml}$), whereas the weakest dependence on shear stress occurs with the highest density of L-selectin (2000 sites/ μm^2 , 5.0 $\mu\text{g/ml}$).

Above 2.05 dynes/ cm^2 , the rolling velocity of sLe^x-coated spheres on all L-selectin densities does not increase monotonically with shear stress. We have carefully repeated these experiments and have checked our flow chamber calibrations thoroughly. This plateau in rolling velocities may represent yet another unusual L-selectin-mediated trend whose mechanism is unclear. However, it should be noted that these results do not affect the main conclusions of this paper, which are drawn from our data at wall shear stresses below 2 dynes/ cm^2 .

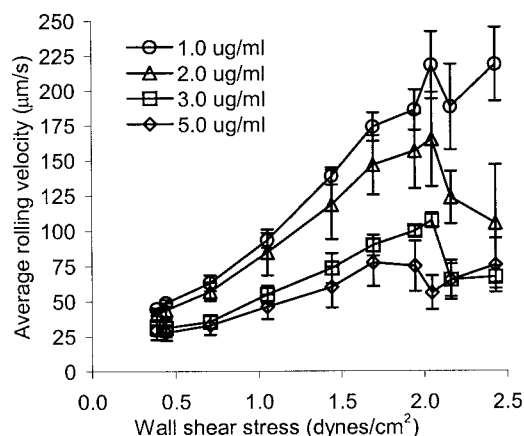


FIGURE 5 Average rolling velocity of sLe^x-coated microspheres over human L-selectin as a function of wall shear stress and L-selectin site density. Rolling velocity increases with increases in wall shear stress or decreases in L-selectin site density. A concentration of 1.0, 2.0, 3.0, or 5.0 $\mu\text{g/ml}$ L-selectin-IgG was added to slides during incubation. Microspheres were prepared using 1.0 $\mu\text{g/ml}$ biotinylated sLe^x. Mean rolling velocities are the result of averaging from four to six independent experiments. Error bars are 95% confidence intervals.

Effect of L-selectin site density changes and wall shear stress on rolling flux

In order to ascertain if the shear threshold effect is present in the sLe^x/human L-selectin cell-free system, the rolling flux of sLe^x microspheres was determined at each wall shear stress for saturating and reduced levels of L-selectin (Fig. 6, with error bars representing 95% confidence intervals). On 5.0 and 3.0 $\mu\text{g/ml}$ L-selectin (Fig. 6, A and B, densities 2000 and 1500 sites/ μm^2 , respectively), rolling flux decreases with increasing wall shear stress above 0.45 dynes/ cm^2 . Below 0.45 dynes/ cm^2 , rolling flux appears to remain stable or increase slightly, but differences are not significant (at a 95% confidence level). In contrast, on both 2.0 and 1.0 $\mu\text{g/ml}$ L-selectin (Fig. 6, C and D, densities 1100 and 800 sites/ μm^2 , respectively), rolling flux increases with increases in wall shear stress up to 0.7 dynes/ cm^2 , then decreases for wall shear stresses greater than 0.7 dynes/ cm^2 . For both 2.0 and 1.0 $\mu\text{g/ml}$ L-selectin, the rolling flux observed at 0.4 dynes/ cm^2 is significantly lower than that measured at 0.7 dynes/ cm^2 (at a 95% confidence level).

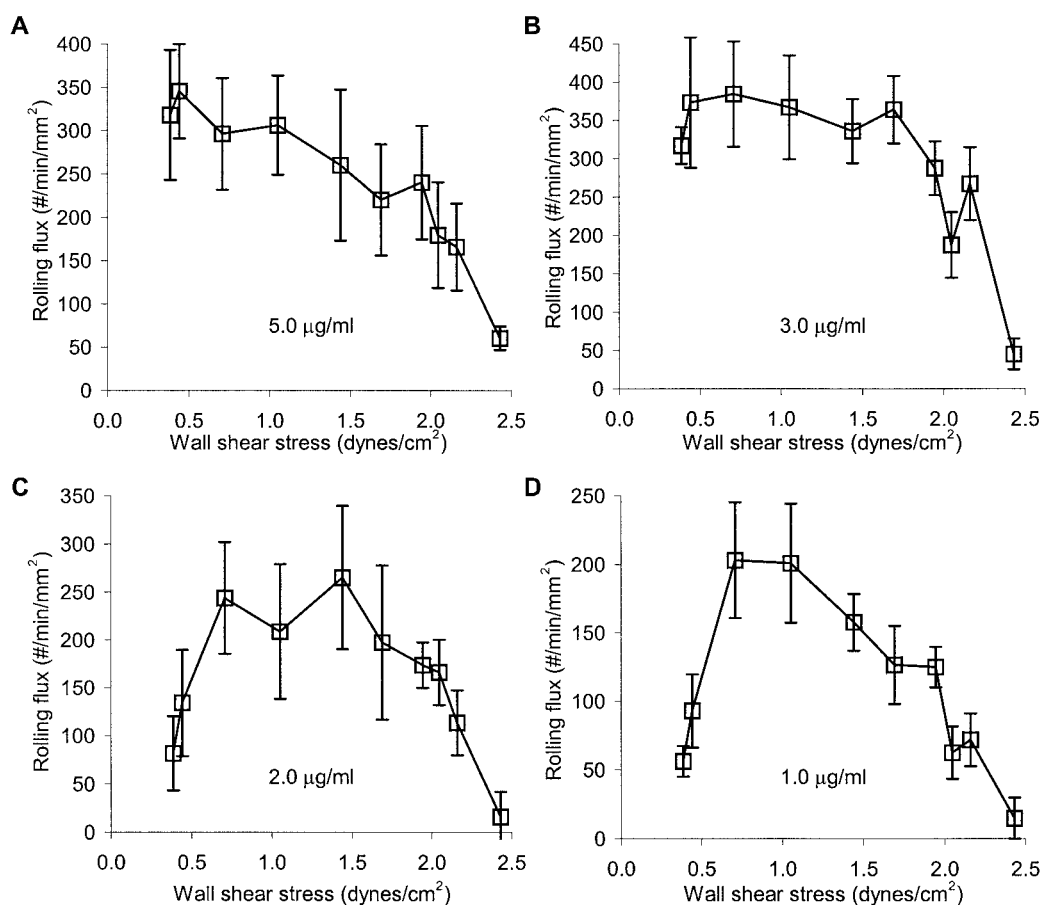


FIGURE 6 Rolling flux of sLe^x coated microspheres on human L-selectin substrates as a function of wall shear stress and L-selectin site density. Rolling flux decreases with decreases in L-selectin site density and is maximal at 0.7 dynes/cm² at reduced L-selectin site densities. However, a minimum shear requirement for rolling is not evident at higher site densities of L-selectin. (A) Substrates were prepared by incubating with 5.0 μg/ml L-selectin-IgG. (B) 3.0 μg/ml L-selectin. (C) 2.0 μg/ml L-selectin. (D) 1.0 μg/ml L-selectin. Microspheres were prepared using 1.0 μg/ml biotinylated sLe^x. Mean rolling velocities are the result of averaging from four to six independent experiments. Error bars are 95% confidence intervals.

These results reveal the presence of the shear threshold effect with the sLe^x/human L-selectin cell-free system.

Decreases in L-selectin density decrease the average rolling fluxes across all the wall shear stresses observed. This decrease in rolling flux is greatest at the lowest and highest wall shear stresses. With a 60% decrease in L-selectin density (from 2000 to 800 sites/μm²), average rolling flux decreases 75% at 2.45 dynes/cm² and 80% at 0.4 dynes/cm². However, at a middle range shear stress of 1.05 dynes/cm², average rolling flux decreases only 35% for the same decrease in L-selectin density.

Comparison of sLe^x interactions with mouse and human L-selectin

Average rolling velocities on 2.5 μg/ml (~1400 sites/μm²) mouse L-selectin range from 28 μm/s at 0.6 dynes/cm² to 139 μm/s at 2.2 dynes/cm². On 3.0 μg/ml (~1500 sites/μm²) human L-selectin, mean rolling velocities are very similar, ranging from 35 to 107 μm/s over the same range of

shear rates. Also, the shear stress at which the rolling flux is maximum is similar, occurring at 1.0 dyne/cm² on 2.5 μg/ml mouse L-selectin, compared to 0.7 dyne/cm² on 2.0 and 1.0 μg/ml human L-selectin. In vitro similarities between mouse and human L-selectin have also been observed by comparing the L-selectin-mediated rolling of human and mouse leukocytes over murine GlyCAM-1 (Dwir et al., 1998). A significant difference we find between mouse and human L-selectin is that the plateau of rolling velocities with increases in wall shear stress above 2.05 dynes/cm² on human L-selectin is not seen on mouse L-selectin. Also, rolling fluxes on mouse L-selectin are higher than on human L-selectin across all the wall shear stresses observed. The reasons for these differences between human and mouse L-selectin is unclear.

DISCUSSION

Using the cell-free system we have previously described (Brunk et al., 1996; Brunk and Hammer, 1997), we investigated the interaction of sLe^x-coated microspheres with

L-selectin substrates. This interaction is specific, as it is completely inhibited by the addition of EDTA, fucoidan, or an antibody to L-selectin. In addition, we have accurately reproduced the general trends of L-selectin-mediated rolling dynamics that have been observed for in vitro cellular systems, including the dependence of rolling velocity and rolling flux on wall shear stress and L-selectin site density. It appears that placing the L-selectin in the stationary rather than the fluid phase has little or no effect on the properties of L-selectin-mediated rolling as confirmed by others (Fuhlbrigge et al., 1996; Alon et al., 1998). The sLe^x/L-selectin cell-free system also reveals the shear threshold effect and shows a dependence of the shear threshold requirement on L-selectin site density.

The surface densities of sLe^x on the microspheres and L-selectin on the substrates used in our experiments are physiologically relevant. The estimated site densities of human L-selectin on our substrates ranges from 800 to 2000 sites/ μm^2 (1.0 to 5.0 $\mu\text{g/ml}$ incubation concentration), while the estimated site densities of sLe^x on our microspheres is 90 μm^{-2} . On neutrophils, assuming a microvillous density of 1.1 μm^{-2} and 260 L-selectin molecules per microvillous (Chen and Springer, 1999), one can calculate an effective L-selectin surface density of ~ 285 sites/ μm^2 , which is three- to sevenfold lower than the density of L-selectin on our substrates. There is approximately threefold more sLe^x than L-selectin on the surface of neutrophils, as determined by fluorescent antibody staining (Fuhlbrigge et al., 1996), resulting in a sLe^x surface density of ~ 850 μm^{-2} , which is ninefold greater than the density of sLe^x on our microspheres. It is important to note that the site densities we measured may overestimate the actual number of sLe^x or L-selectin molecules oriented in the correct position for binding ligand. However, the surface densities of sLe^x on microspheres and L-selectin on substrates are within an order of magnitude of the sLe^x and L-selectin surface densities on neutrophils, allowing us to make useful comparisons to cellular systems.

It is useful to compare our findings with those of other studies examining the interaction of sLe^x with L-selectin. In static assays, Berg and coworkers (1992) found that L1-2 cells transfected with L-selectin bound neoglycoproteins containing sLe^x, whereas Foxall and coworkers (1992) and Galustian and colleagues (1997) observed that L-selectin chimera bound sLe^x containing glycolipids. In a flow chamber assay, Alon and colleagues (1995) found that Jurkat T cells expressing L-selectin tethered to and rolled on glycolipid substrates bearing sLe^x. Our results are the first to show that sLe^x can mediate rolling interactions with L-selectin under shear flow in a cell-free system. Differences in the rolling behavior of sLe^x microspheres on mouse versus human L-selectin may be due to mouse L-selectin displaying slightly modified binding epitopes when compared to human L-selectin. It has been shown previously that very fine differences in carbohydrate chemistry can

greatly affect interactions between the selectins and their ligands (Pouyani and Seed, 1995; Brunk and Hammer, 1997). Our work strongly suggests that sLe^x is a minimum functional binding element required for L-selectin-mediated rolling.

The average and instantaneous rolling velocity and rolling flux of sLe^x microspheres on L-selectin varies with changes in L-selectin site density. As the site density of L-selectin on the substrate decreases, rolling becomes faster and less smooth, with larger velocity fluctuations. With additional decreases in L-selectin site density, the tethering limit is eventually reached, corresponding to a lack of stable adhesion. Conversely, the mean rolling fluxes on L-selectin decrease with decreasing L-selectin site density. Our results agree well with those of Puri and colleagues (1997) who also showed that neutrophil rolling velocities and tethering and rolling fluxes over CD34 substrates vary similarly with CD34 site density changes and Alon and coworkers (1995) who found that the rolling velocity of Jurkat T-cells expressing L-selectin increases with decreasing site densities of sLe^x glycolipids.

We find that sLe^x microsphere rolling velocities over saturating concentrations of L-selectin are an order of magnitude faster than those previously reported for sLe^x microspheres rolling over saturated E-selectin substrates (Brunk et al., 1996; Brunk and Hammer, 1997). Faster rolling velocities observed for sLe^x microspheres over L-selectin suggest that the kinetic dissociation rate (off rate) is higher between sLe^x and L-selectin than between sLe^x and E-selectin. This is supported by tethering experiments in which bond dissociation rates are seven- to tenfold more rapid for L-selectin than for E- and P-selectin (Alon et al., 1997).

The rolling of sLe^x microspheres over L-selectin exhibits a shear threshold effect that disappears with increasing L-selectin site density. The rolling flux of sLe^x microspheres over 1.0 and 2.0 $\mu\text{g/ml}$ (800 and 1100 sites/ μm^2 , respectively) human L-selectin substrates is greatest at a wall shear stress of 0.7 dynes/ cm^2 . Similarly, a maximum in rolling flux occurs at 1.0 dynes/ cm^2 on 2.5 $\mu\text{g/ml}$ (~ 1400 sites/ μm^2) mouse L-selectin. However, a shear threshold is not evident on higher site densities of human L-selectin (3.0 and 5.0 $\mu\text{g/ml}$, 1500 and 2000 sites/ μm^2 , respectively). The shear threshold behavior exhibited by sLe^x microspheres over reduced concentrations of L-selectin has also been observed by others in cellular systems. T lymphocytes rolling over PNA^d or CD34 substrates have been shown to roll maximally at a wall shear stress of 0.7 dynes/ cm^2 (Finger et al., 1996) or 0.8 dynes/ cm^2 (Lawrence et al., 1997; Puri et al., 1998). The shear stress range over which the shear threshold occurs in our experiments (0.7 to 1.0 dynes/ cm^2) is the same as seen with cells in these experiments. In these cellular experiments, the shear threshold was preserved even when the site density of CD34 was increased nearly threefold (Finger et al., 1996) or sixfold (Puri et al., 1998).

These findings contrast with our results, which show the disappearance of the shear threshold effect with a 2.5-fold increase in L-selectin site density. However, the specified site densities of CD34 ($\sim 100\text{--}300$ sites/ μm^2 and $50\text{--}300$ sites/ μm^2) used in these studies may be too low to eliminate the shear threshold effect. As a comparison, the shear threshold effect we observe only disappears when the L-selectin surface densities reach $\sim 1500/\mu\text{m}^2$ ($3.0\ \mu\text{g}/\text{ml}$ bulk concentration). Differences in the density of molecules that can support rolling and the shear threshold between cellular and cell-free experiments may reflect differences between microsphere and cellular topography. It would be interesting to determine if increasing the CD34 surface density further would eliminate the shear threshold effect observed for lymphocyte rolling. Also, it is possible that a shear threshold does in fact exist in our system at the higher L-selectin site densities tested, but falls below the range of shear stresses observed. Alon and colleagues (1998) observed that neutrophils stopped rolling on L-selectin substrates at wall shear stresses below $0.4\ \text{dynes}/\text{cm}^2$, which coincides with the lowest wall shear stress we examined. Thus, we conclude that the shear threshold effect is either shifted to a lower shear stress or eliminated completely by an increase in L-selectin site density. However, the most remarkable finding is that one can recreate the shear threshold in a cell-free system and that it occurs at a very similar wall shear stress as is seen in cellular systems, arguing strongly that the primary cause of the shear threshold effect is molecular, and that cellular features may modulate, but do not control the effect.

Mechanisms that have been proposed to explain the shear threshold effect vary widely. Puri et al. (1998) propose that the elasticity of mucin-like regions of L-selectin ligands affects the mechanical properties of the L-selectin-ligand bond and may give rise to the shear threshold effect, whereas Alon and colleagues (1997) suggest that shear flow may elongate mucin molecules and better expose the carbohydrates they bear for recognition by selectins. Others have proposed that fluid shear may deform the cell slightly after the first bond cluster forms, thereby increasing the time and contact area to favor further bond formation (Lawrence et al., 1997; Alon et al., 1997). Most recently, Chen and Springer (1999) have proposed that a minimum force or velocity with which selectins contact their ligands is necessary to overcome or penetrate a repulsive barrier, proposed to be an electrostatic and steric “cloud” around the mucin-like domain of selectin ligands, and promote bond formation. With the use of a cell-free system such as ours, cell deformation can be discounted as an explanation for the shear requirement for rolling on selectins, since the polystyrene latex microspheres we use are much more rigid than cells, yet still exhibit the shear threshold effect. The steric “cloud” hypothesis and explanations based on the elasticity and elongation of mucins can also be ruled out, since our

system utilizes sLe^x, which by itself is not a mucin, as the sole ligand for L-selectin.

Our experiments indicate that shear threshold effect observed is a function of molecular binding. The mechanism we propose to explain the shear threshold effect originates from a recent study by Chang and Hammer (1999), which models the overall rate of reaction of species that are bound to surfaces under relative motion. They show that the rate of collision between receptor and ligand increases with shear rate, and the encounter duration decreases. Depending on the rate of bimolecular reaction, increases in shear rate can increase adhesion (by increasing the encounter frequency) or decrease adhesion (due to the decreased encounter time). The shear threshold would occur at shear rates at which these effects are counterbalanced. An increase in tethering frequency (Alon et al., 1997) and bond number (Chen and Springer, 1999) with increases in shear stress up to $0.7\ \text{dynes}/\text{cm}^2$ has been measured experimentally for neutrophils rolling over PNAd. Our suggested mechanism also helps to explain why the shear threshold effect disappears with increases in the site density of L-selectin. At very high site densities of either receptor or ligand, the collision rate is very high, even at low shear stresses, resulting in increased probability of bond formation. We hope to further demonstrate the mechanism of the shear threshold effect through adhesive dynamics simulations (Hammer and Apte, 1992) in the future.

The results presented in this work suggest a number of future directions for L-selectin research using a cell-free system. It would be interesting to place L-selectin on microspheres and ascertain if this physical modification has an effect on its interaction with sLe^x. Also, as sulfation of sLe^x at specific positions has increased the ability of sLe^x to bind to L-selectin in static assays (Scudder et al., 1994) and also increased the rolling and tethering frequency of lymphocytes to GlyCAM-1 (Tangemann et al., 1999), it would be useful to see how a sulfated-sLe^x molecule attached to microspheres would interact with L-selectin substrates under flow. In addition, multivalency of L-selectin has been shown to increase the avidity of binding to sLe^x in static assays (Galustian et al., 1997). Therefore, it would be interesting to contrast our results to those using a univalent form of sLe^x. Finally, because chemical modification of CD34 substrates with periodate has been shown to eliminate the shear threshold effect with lymphocyte mediated rolling (Puri et al., 1998), it would be useful to study the effect of this enzyme on sLe^x-mediated rolling with L-selectin.

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